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Adhered Membranes Retaining Porosity and Biological Activity

This application claims benefit of priority to U.S. Provisional Application No. 60/460,079, filed April 2, 2003.

1. Field of the Invention

The present invention relates to a high density lipoprotein (HDL)-associated cholesterol assay pad, a method for using said pad, and a diagnostic assay device for carrying out the method.

2. Background of the Invention

The amount of cholesterol present in the blood is known to be related to the risk of coronary artery disease. Cholesterol circulates in the blood predominantly in protein-bound form. The proteins which transport cholesterol are the lipoproteins, which are subdivided into three classes based on their density. The very-low density lipoproteins (VLDL) are triglyceride-rich lipoproteins which are synthesized in the liver and ultimately converted to low-density lipoproteins (LDL), which transport most of the plasma cholesterol in humans. The high-density lipoproteins (HDL) are lipoproteins which are involved in the catabolism of triglyceride-rich lipoproteins, and in the removal of cholesterol from peripheral tissues and transport to the liver. An inverse relationship between serum HDL levels and risk of coronary disease has been established. In particular, if the proportion of serum cholesterol associated with HDL is low, the risk of coronary disease is increased.

In view of the importance of relative serum cholesterol levels in risk assessment and management of atherogenic disease, considerable effort has been spent screening large populations of both normal and high-risk individuals for serum levels of HDL, LDL, as well as total cholesterol and triglycerides. The effectiveness of treatments of high-risk individuals has been monitored by regular testing of serum levels of cholesterol in the various lipoprotein compartments.

One method for specific HDL cholesterol testing is based on the selective precipitation of non-HDL lipoproteins in serum by a polyanionic compound, such as dextran sulfate, heparin, or phosphotungstate, typically in the presence of a group II

cation, such as Mg^{2+}, Mn^{2+}, or Ca^{2+}. The specificity and degree of precipitation are dependent on a variety of factors, including the type and concentration of the precipitating reagent. In general, the order of precipitation of serum cholesterol particles, with increasing concentration of polyanion, is VLDL, LDL, and HDL. HDL usually remains soluble at concentrations of heparin or dextran sulfate which completely precipitate lower density particles, although minor apoE species of HDL may be co-precipitated with lower density particles. By selective precipitation of lower density particles, HDL serum cholesterol levels can be determined.

In a typical lipid assay procedure, a small volume of blood is drawn and centrifuged to produce a clear plasma or serum sample fluid. The sample fluid is then aliquoted into several assay tubes, for determination of (a) total serum cholesterol, (b) triglycerides, and (c) HDL cholesterol. The HDL sample is precipitated, as above, and the lower density particles are removed by filtration or centrifugation prior to cholesterol detection. The samples are then reacted with an enzyme mix containing cholesterol esterase, cholesterol oxidase, peroxidase, and a dye which can be oxidized to a distinctly colored product in the presence of H_2O_2. The tubes may be read spectrophotometrically, and the desired total, HDL and LDL cholesterol values determined.

Despite the accuracy and reliability which can be achieved with the liquid-phase cholesterol assay just described, the assay has a number of limitations for use in widespread screening. First, the method uses a venous blood sample, requiring a trained technician to draw and fractionate the blood sample, and aliquot the treated blood to individual assay tubes. At least one of the sample tubes (for HDL determination) must be treated with a precipitating agent and further processed to remove precipitated material. Although some of these procedures can be automated, analytical machines designed for this purpose are expensive and not widely available outside of large hospitals.

Co-owned U.S. Patent Nos. 5,213,964, 5,213,965, 5,316,196 and 5,451,370, each of which is incorporated herein by reference, disclose methods and assay devices which substantially overcome many of the above-mentioned problems associated with liquid-assay procedures for measuring serum cholesterol levels. In one embodiment, the device is designed for measuring the concentration of HDL-

associated cholesterol in a blood sample also containing LDL and VLDL particles. The device includes a sieving matrix capable of separating soluble and precipitated lipoproteins as a fluid sample migrates through the matrix. A reservoir associated with the matrix is designed to release a soluble precipitating agent, for selectively precipitating LDL and VLDL, as fluid sample is drawn into and through the matrix. This allows HDL separation from the precipitated lipoproteins, based on faster HDL migration through the sieving matrix. The fluid sample, thus depleted of non-HDL lipoproteins, then is transferred to a test surface where it is assayed for cholesterol.

The above-referenced devices, while representing an advance over liquid-phase assays, present the possibility of contamination of the flow transport path with the precipitating reagents. Such reagents could interfere with HDL quantification, or with other assay chemistry taking place on other regions of a multi-assay device. The present invention addresses and overcomes these problems.

Further methods and devices for measuring HDL cholesterol in blood samples are disclosed in EP 0408223 and EP 0415298 (Rittersdorf *et al.*), which describe a continuous assay method carried out on a test strip comprising the following steps and corresponding elements. The blood sample is applied to a separation layer for separating cellular blood constituents. Driven by capillary forces or gravity, the sample flows through a further carrier containing soluble precipitating agents, which, after dissolving in the serum sample, precipitate non-HDL lipoproteins contained in the sample. In a further carrier, the precipitated constituents, above, are filtered from the serum sample to prevent their interference with later HDL quantification. In the same carrier, the sample is transported to a position adjacent the HDL-quantification carrier, and is stored until the HDL quantification step is to be started. Finally, the sample is transferred to an HDL quantification layer, where HDL cholesterol in the serum sample is quantified by an enzymatic reaction.

A disadvantage of this assay design is that the carrier functioning as a reservoir allows migration of the precipitated constituents or soluble reagents into the sample, which can interfere with HDL quantification. In addition, during the storage of the serum sample, HDL can be trapped by adhering to the carrier fibers, precipitating reagents can cause further undesired reactions, and the

carrier can become clogged by the drying serum sample.

U.S. Patent No. 5,135,716 (Thakore) discloses additional devices and methods for HDL quantification in a blood fluid sample. In these devices, the fluid sample flows continuously, though an unbroken path, from an inlet well to a carrier for HDL quantification. Accordingly, the ability to control sample volume entering the HDL test carrier, and to control environmental conditions for the HDL assay, is limited. Nor do the devices provide for simultaneous assay of various analytes from a single fluid sample.

It is therefore the object of the present invention to provide a HDL assay device which overcomes the above-noted prior art disadvantages.

Summary of the Invention

In one aspect, the invention includes an assay pad formed by joining a HDL test pad and a reagent pad where the assay pad retains porosity sufficient for the sample to pass through the reagent pad to the HDL test pad. The assay pad further retains the biological activity of the reagents on the reagent pad and HDL test pad.

In another aspect, the invention includes an assay device and method for using the device for measuring serum cholesterol associated with high-density lipoproteins (HDL) in a blood fluid sample containing lipoproteins other than HDLs. The device includes a sample distribution array, a HDL test pad in which HDL concentration can be assayed; and a reagent pad containing a binding reagent effective to selectively bind and remove non-HDLs from the fluid sample. The HDL test pad and reagent pad are joined by an adhesive layer or by heating the pads.

In yet another aspect, the invention provides methods of preparing the joined HDL test pad and reagent pad.

Brief Description of the Drawings

Fig. 1 is a side view of one embodiment of the multi-analyte assay device;

Fig. 2 is a perspective view, in exploded form, of a multi-analyte assay device constructed in accordance with one embodiment of the invention; and

Fig. 3 is a partial side view of the multi-analyte assay device in accord with one embodiment of the invention.

Detailed Description of the Invention

I. Definitions

The terms below have the following meanings unless indicated otherwise.

An element is in "fluid communication" with another element when a fluid is able to travel from one element to the other via capillary action and/or gravity. The elements do not need to be in direct contact; *i.e.*, other elements through which said fluid can pass may be intervening.

A "pad", as used in the context of a "sample distribution pad", "test pad", "HDL test pad", and "reagent pad" intends a thin, flat mat or cushion, or a piece of absorbent material. Pads may be comprised of any material, such as a porous membrane or fibrous strip, which can contain impregnated or immobilized reagents and through which fluid can move via capillary action and/or gravity.

II. Assay Device

The device in accord with the invention is illustrated in Figs. 1-3, which will be discussed below. For convenience, similar element numbering is retained in all Figs. 1-3 to identify like structural features. The device is designed particularly for determining serum cholesterol associated with HDL (also referred to as HDL-associated cholesterol or simply HDL cholesterol) using a small volume of blood or serum sample, typically between 10-50 μL. Other assays, such as total cholesterol, triglyceride, glucose, alanine aminotransferase level (ALT), aspartate amino transferase (AST), Blood Urea Nitrogen (BUN), or creatinine can be determined simultaneously from the same sample. Determination of HDL-associated cholesterol may also be referred to simply as determination of HDL or an HDL assay.

With initial reference to Figs. 1-3, various embodiments of the multiple-analyte assay device are illustrated, with Fig. 2 shown in exploded format. As best seen in Fig. 1, the multiple-analyte assay device **14** includes a main body or support **15** which defines a well **16** dimensioned and sized to receive a quantity of a blood sample, typically between about 25-50 μL. The well may be in fluid contact with an optional sieving pad **22**, which may be carried in a notched region **20** formed in the upper edge of the support. The fluid contact may be direct, or as in the device

shown in Fig. 1, provided by a capillary conduit **18** formed in the plate at the base of the well. The support is preferably a plastic plate, with the well, notched region and/or capillary formed by standard molding or machining methods.

Sieving pad **22** carried in region **20** functions to partially remove large particulate matter (including blood cells) as the sample migrates through the pad matrix in a bottom-to-top direction as shown in the figure. Pad **22** is preferably formed of a glass fibrous matrix of material designed to draw aqueous fluid by surface wetting, and to retard the movement of blood cells as the blood sample is drawn through the matrix. One exemplary pad is a glass fiber filter, such as a GF/D, PD008, or F145-02 filter available from Whatman, having a packing density of about 0.16 g/cm^3, and a thickness of about 1 mm. The pad is dimensioned to absorb a defined volume of sample fluid, preferably between about 15-25 μL. Sieving pad **22** may additionally contain red blood cell capture reagents, such as lectins, antibodies specific for red blood cell surface membrane proteins, thrombin, or ion exchange agents. In one embodiment, the pad may contain reagents for removal of non-HDL lipoproteins, as described further below.

The sample contacts an elongate strip or sample distribution array **26**. The sieving pad **22** may be in fluid contact with array **26** between the well **16** and the array **26**. In a preferred embodiment, array **26** is formed of three or more separate membranes in fluid communication. In an embodiment where array **26** is formed of three membranes in fluid communication, as seen in Fig. 2, central sample-application membrane **28** distributes sample fluid to sample-collection membranes **30** and **32**. The array **26** may further comprise one or more reagent membranes, not shown, disposed between sample-application membrane **28** and sample-collection membrane **30**. The reagent membrane may contain one or more reagents. In one embodiment, the reagent membrane may contain one or more reagents for selectively removing LDL and VLDL as described further below. In another embodiment, reagent membrane may function as a wick to draw sample for multiple tests. Array **26** may also be supported by foam cushions **27** or other supports, as shown in Fig. 2. Array **26** is preferably multiple membranes formed of a matrix of glass fibers. The packing density and thickness of the matrix are such as to absorb and distribute volumes of sample fluid, *e.g.*, 10-25 μL, supplied to the

sample-application membrane and to the sample-collection membranes. The matrix has a preferred packing density between about 0.16 g/cm^3 and 4.0 g/cm^3. One exemplary strip material is a F-165-25A glass fiber filter available from Whatman, having a packing density of about 0.2 gm/cm^3 and a thickness of about 0.12 mm.

The device further includes four or more test pads **64**, **66**, **68**, and **70**, which are wettable, absorbent reaction test pads. Each test pad used in a particular assay contains analyte-dependent reagents effective to produce an analyte-dependent change in the pad which can be detected in a known manner, as described further below. All or any integral subset of the test pads may be employed in a particular assay.

Desirably, the test pads are porous polymer membranes, preferably having a thickness of about 100-150 μm and side dimensions of about 3 mm. The absorption volume of each pad is preferably between about 0.5-1.0 μL. In one embodiment, some or all of the reaction pads are asymmetric membranes; that is, membranes having a porosity gradient across the thickness of the membrane.

In one embodiment, test pad **64** is a HDL test pad containing reagents effective to produce a change in the pad in response to the presence of HDL. In another embodiment, the HDL test pad **64** is also a polymeric membrane, containing reagents for assaying HDL level. One exemplary strip material is a BTS-83 asymmetric polysulfone membrane available from Pall Corporation (East Hills, NY). If desired, HDL assay reagents, such as peroxidase, may be immobilized to the test pad membrane, according to well known methods for enzyme immobilization. (See *e.g.* U.S. Patent No. 4,999,287; U.S. Patent No. 5,419,902; Blum, L.J. *et al., Anal. Lett.* 20(2):317-26 (1987); Kiang, S.W. *et al., Clin. Chem.* 22(8):1378-82 (1976); Guilbault, G.G., Ed., *Modern Monographs in Analytical Chemistry, Vol. 2: Analytical Uses of Immobilized Enzymes* (1984); Torchilin, V.P., *Progress in Clinical Biochemistry and Medicine, Vol. 11: Immobilized Enzymes in Medicine* (1991)).

A reagent pad **74** contacts the HDL test pad **64** and contains the chemicals for precipitation of non-HDL cholesterol. In one embodiment, the reagent pad is the same material as the HDL test pad, *i.e.* an asymmetric polysulfone membrane. In a preferred embodiment, reagent pad **74** is composed of a porous polymeric

membrane, having pore sizes of about 1μm or less. In the device shown in Fig. 1, reagent pad **74** consists of a single membrane, however, the invention also contemplates the use of multiple stacked membranes, *i.e.* up to about six, where at least one and preferably each membrane contains reagents for binding non-HDL lipoproteins.

The reagent pad preferably has a thickness of about 100-150 μm, side dimensions of about 3 x 6 mm, and an absorption volume of about 0.5-1.0 μL. It contains at least one reagent effective to selectively remove LDL and VLDL particles from the fluid sample. The reagent may be, for example, an antibody, or preferably a polyanionic LDL- and VLDL binding reagent. Such reagents, which are known in the art, include sulfonated polysaccharides, heparin, and phosphotungstate, in the presence or absence of a group II cation, such as Mg^{2+}, Mn^{2+}, or Ca^{2+}. A preferred reagent is a sulfonated polysaccharide, such as dextran sulfate, having a typical molecular weight of 50,000 to 500,000 daltons, optionally in combination with magnesium acetate or chloride, optionally buffered to maintain neutral pH. The reagent may be an immobilized reagent effective to bind, and remove from the fluid sample, non-HDL lipoproteins. The reagent pad is effective to entrap bound non-HDL lipoproteins within the reagent pad and prevent them from entering HDL test pad **64.** In one embodiment, a reagent, such as catalase, which is effective to decompose any generated hydrogen peroxide that might diffuse downward from test pad **64**, may be immobilized in reagent pad **74**.

HDL test pad **64** and reagent pad **74** are adhered together using heat alone, as described in Example 6, or an acrylic acid copolymer that can be melted as a heat-activated adhesive, as described in Examples 1-3. The combined HDL test pad and reagent pad is called the assay pad.

Device **14** also includes a reaction bar **60**, which is an elongate support, which may be transparent or have windows, *e.g.* window **76** (Fig. 2). The window(s) allow the test pads **64**, **66**, **68**, and **70** to be viewed through the support. These windows may be transparent materials or simply openings in the support. The test pads and assay pad are attached to the reaction bar by a transparent or translucent adhesive material, by sonic welding, or other suitable bonding methods.

The reaction bar is mounted on support **15** by mounting means effective to (a) maintain the device in a sample-distribution position, wherein the test pads and assay pad are spaced apart from the sample distribution array, the sieving pad or the well, and to (b) transfer the device to a test position, where the test pads and assay pad are in fluid communication with the sample distribution array, the sieving pad and the well. The mounting means can also be used to break such fluid communication after a desired amount of sample has entered the assay pad and/or the test pads, and/or after a determined contact time, by transferring the device from the test position to a position in which the assay pad and/or the test pads are not in fluid communication with the sample well (which may be the same as the "sample-distribution" position). Such transferring can be controlled by monitoring the reflectance at the top surface of the test pads, which reflects extent of wetting, as described in co-owned U.S. Patent No. 5,114,350, the content of which is incorporated by reference herein. Alternatively, when the absorption capacity and rate of sample uptake of the pad materials are known, the quantity of sample can be controlled with sufficient accuracy simply by using a predetermined contact time.

The mounting means can include, for example, a pair of resilient members, such as elastomeric blocks **71**, **72**, which act to bias the assay pad toward a non-transfer or sample-distribution position, at which the pads are spaced apart from the sample distribution array, the sieving pad, the capillary conduit or the sample well. By compression or release of the resilient members, fluid communication between the assay pad can be selectively established and separated, as shown in Fig. 3. The fluid communication may be via direct contact or through an intermediate element. The support blocks could be compressed by means of springs or a piston-like action. Alternatively, external mechanical devices could engage the main body **15** and/or reaction bar **60** and move one towards the other. Such devices may include conventional components such as clamps, pistons, stepper motors, worm gears, or the like. An exemplary system is the Cholestech LDX® Analyzer, a self-contained, automated analyzer advantageous for use with assay devices such as described herein.

In a further embodiment, the HDL test pad comprises a biosensor, as described, for example, in PCT Publication. No. WO 99/58966 (Dobson *et al.*),

which is incorporated herein by reference. This document discloses a microscale biosensor device, comprising a conducting surface, a layer of dielectric material overlying the conducting surface, and a plurality of pores extending through the dielectric layer. Each of the pores contains a biopolymer in contact with the conducting surface, and can act as a microelectrode, converting a chemical response into an electrical signal. In use, a fluid containing an analyte to be assayed is applied to the pores so as to be in contact with the biopolymer. In the present HDL assay device, this is achieved when the HDL test pad **64** is in fluid communication with the sample, *i.e.* the test position as shown in Fig. 3.

The biopolymer within the microelectrode pores is typically an enzyme, such as, for the measurement of HDL-associated cholesterol, cholesterol oxidase. Cholesterol is oxidized by cholesterol oxidase to the corresponding ketone, liberating hydrogen peroxide, which can then be converted to water and oxygen by the enzyme peroxidase. Either oxygen or hydrogen peroxide can then be measured electrochemically. Electrochemical methods that may be used include amperometric methods, as in the Clark oxygen electrode, which measures current produced by reduction of oxygen or oxidation of hydrogen peroxide, or voltammetric methods. The use of cyclic voltammetry at microelectrodes has been described for measurement of various analytes (see *e.g.* R.J. Forster, *Chem. Soc. Rev.* 289-297 (1994)), such as dopamine (Pihel *et al., Anal. Chem.* **68**(13):2084-9 (1996)) and fullerenes (Soucaze-Guillous *et al., Anal. Chem.* **65**(6):669-72 (1993)) as well as hydrogen peroxide (Horrocks *et al., Anal. Chem.* **65**(24):3605-14 (1993); Nowall *et al., Electroanalysis* **9**(2):102-9 (1997); Dequaire *et al., J. Am. Chem. Soc.* **124**(2):240-53 (2002).

III. Preparation of the Assay Pad Using an Adhesive Layer

As seen in Fig. 1, in one embodiment, the assay pad is prepared by forming an adhesive layer **80** between the reagent pad **74** and the HDL test pad **64**, as described in Examples 1-3. Joined by adhesive layer **80**, reagent pad **74** and HDL test pad **64** form a composite structure referred to as the assay pad. It will be appreciated that the adhesive layer need only be sufficient to secure the opposing

pads, *e.g.*, by a peripheral seal, or can extend over the entire surfaces of the opposing pads.

The adhesive layer is preferably formed of an acrylic acid copolymer. The copolymer will typically, but not necessarily, have a melting point below the denaturing temperature of the HDL assay reagents used or below a temperature that is otherwise damaging to the HDL assay reagents. One exemplary copolymer is an Ethylene Acrylic Acid copolymer (EAA). EAA is available as different particle size dispersions from Michelman (Cincinnati OH, P/N MP 4990R with a melting point of about 75° C). One preferred particle size is 30-500 nm, with an average particle size of about 90 nm. Other polymers, including polyethylene glycol, polyethylene terephthalate, and polyvinyl alcohol, may also find use as the adhesive layer. These copolymers may be cross-linked and may further be copolymerized with a second, different polymer. In another embodiment, the adhesive layer is formed of a pressure sensitive adhesive or a wax emulsion. In yet another embodiment, the adhesive layer is formed of hot melt adhesives as described in U.S. Patent No. 6,596,112, which is incorporated herein by reference. It will be appreciated that any substance may be used for the adhesive layer where the assay pad retains a suitable porosity for the sample to flow from the reagent pad to the HDL test pad.

The composite assay pad is typically prepared by dissolving the copolymer in a suitable solvent, often an aqueous-based solution. A layer of the copolymer emulsion or solution is applied to the reagent pad **74**. Any suitable method for coating the pad may be utilized, including dipping the pads in the emulsion or solution, spray coating the pad or applying the polymer solution to the pad with a wick. The pad may be coated on one side only or on both sides. The pad is dried for a suitable time, *i.e.* for about 20 minutes at 50° C. The precipitating reagent is then dispensed onto the large pore (dull) side of the asymmetric membrane.

When the reagent pad and HDL test pad are both formed of an asymmetric membrane, the reagent pad and HDL test pad are oriented such that the small pore (shiny) side of the reagent pad contacts the open pore (dull) side of the HDL test pad.

The copolymer adhesive layer between the HDL test pad **64** and the reagent

pad **74** is formed by applying heat and/or pressure to the coated reagent pad **74** and HDL test pad **64**, whereby the copolymer forms a bond with the adjacent pad material. The pads may be heated using any suitable device that provides heat at the required temperature and pressure. A preferred temperature for heating the pads is about 80° C. Any suitable lamination machine may be used.

The concentration of copolymer on the reagent pad must be such that the assay pad retains porosity for the sample to flow from the reagent pad to the HDL test pad. Suitable concentrations of copolymer to provide adhesion are about 4.0% to about 10.0% emulsion. Preferred concentrations of the polymer coating are 4%, 5%, 6%, 7%, 8%, 9% and 10%. One preferred emulsion is a 5.5% EAA emulsion.

The HDL reagent may be dispensed onto HDL test pad **64** by any suitable method before or after adhering the test pad to the reagent pad. The HDL reagent is preferably dispensed onto the test pad after adhering the test pad to the reagent pad.

IV. Preparation of the Assay Pad Using Heat

In one embodiment, the assay pad is prepared by adhering the reagent pad **74** containing reagent to the HDL test pad **64** using heat applied at a temperature sufficient to at least partially melt at least one of the reagent pad or the HDL test pad, as described in Example 6. The reagent pad and HDL pad are heated above about 65° C to about 220° C as described in U.S. Patent No. 6,596,112, which is incorporated herein by reference. In a preferred embodiment, when the reagent pad and HDL test pad are heated above about 165° C, the pads become adhered to each other. In other preferred embodiments, the reagent pad and HDL test pads are heated above about 93° C, 121° C, 148° C, 165° C, 200° C, or 205° C. The pads are oriented as described above. The HDL reagent is dispensed onto the HDL test pad before or after adhering the test pad to the reagent pad. In a preferred embodiment, the HDL reagent is dispensed onto the HDL test pad and/or the chemicals for precipitation of non-HDL cholesterol are dispensed onto the reagent pad after adhering the test pad to the reagent pad. In a particularly preferred embodiment, both the HDL reagent and the chemicals for precipitation are dispensed onto the HDL test pad and the reagent pad, respectively, after the test

pad and the reagent pad are adhered together.

V. Dispensing Reagents onto Assay Pad Using Printing Process

In another embodiment, one or more reagents are applied to opposite sides of a pre-laminated membrane using a printing process that incorporates an engraved Gravure or Anilox cylinder. In this embodiment, less than a saturation volume of the HDL reagent and the precipitation reagent are used. By using non-saturating volumes, the reagents may be confined to their respective membranes. A volume of about 80 ml/1000 inch^2 is a typical saturating volume. Volumes in the range of 25 to 55 ml/1000 inch^2 of reagent are suitable, however, in one embodiment, 20 ml/1000 inch^2 reagent is used. The precipitation reagent may be applied before or after the HDL reagent. In other embodiments of this printing process, other reagents that contain two components that must be kept separate can be applied in like manner as described for the HDL reagents. Alternatively, a single reagent can be applied to a single unlaminated membrane.

VI. Assay Method

In operation, a blood sample is placed into well **16**, and is imbibed through sieving pad **22**, where large particulates, including red blood cells, are removed, and thence into the sample distribution array **26**. In one embodiment, these steps take place while the device is in a "sample-distribution" stage, such that the assay pad is not in fluid communication with the sample distribution array, the sieving pad, the capillary conduit, or the sample well.

The plasma sample travels from sample-application membrane **28** to sample-collection membranes **30** and **32**. When the plasma sample reaches sample-collection membranes, the device is adjusted to a test position, *e.g.* as shown in Fig. 3, preferably by moving reaction bar **60**, to place the assay pad in fluid communication with the sample distribution array. In this position, sample fluid in the sample distribution array is drawn into the reagent pad by capillary flow. The sample fluid is further drawn into the HDL test pad(s) by capillary flow. The reaction bar is held at this position until a desired degree of wetting of the test pad(s) is achieved. The bar is then moved, if desired, to break fluid communication between

the sample distribution array and the assay pad, when a desired amount of sample fluid has entered the assay pad(s), and/or after an appropriate contact time.

Prior to contacting the HDL test pad **64**, sample plasma contacts a precipitating or binding reagent, which is contained in a separate reagent pad **74**, such that non-HDL lipoproteins are bound to the respective carrier. The device is thus effective to remove non-HDL lipoproteins from the serum, while allowing passage of sample fluid containing liquid-phase HDL to HDL test pad **64** with these elements (*e.g.* Fig. 2). One advantage of this embodiment is that the sample distribution array and upstream elements do not contain non-HDL binding reagents; such reagents are present only in reagent pad **74**. Therefore, the possibility of interference from these reagents, in assays of analytes other than HDL, is eliminated.

During operation, in embodiments such as illustrated in Figs. 1-3, as sample fluid passes through the HDL assay path, comprising pads **74** and **64**, its leading edge passes in an upward direction through pad **74**, where non-HDL lipoproteins react and are entrapped, and directly to adjacent test pad **64**, where HDL reacts with the assay reagents therein, for measurement of HDL-associated cholesterol. Further portions of sample continue to be in contact with pad **74** during this time, and proceed from pad **74** to pad **64** in a like manner, until the absorption capacity of pad **64** is reached. Accordingly, quantification of HDL-associated cholesterol in test pad **64** occurs concurrently with the binding reaction taking place in reagent pad **74**. Preferably, the volume of sample fluid transferred to the HDL assay path (comprising pads **74** and **64**) from the sample distribution matrix is equal to or greater than the absorption capacity of test pad **64**, and less than or equal to the combined absorption capacity of test pad **64** and reagent pad **74**.

In these embodiments, when the sample fluid contacts reagent pad **74** containing the binding reagents, the latter is in direct contact with HDL test pad **64**, thus limiting the temporal contact of the blood sample with the binding reagents prior to the HDL assay reaction. Sample preparation and HDL evaluation are thus carried out in separate steps, where sample preparation includes, for example, filtering of cellular blood components and, optionally, temporary storage of the blood sample and adaptation of the blood sample to such test requirements or conditions as temperature, pressure and

environmental atmosphere. Because the temporal contact of the blood sample with the different reagents is reduced, any chemical interference with the HDL evaluation is prevented. If desired, the assay can be interrupted for a desired time after the sample application and removal of cellular components, but prior to contact with binding reagents, *e.g.* to adjust the surrounding atmosphere or adapt the environmental temperature to support the testing. This is accomplished by maintaining the device in the sample-distribution position. To this end, the sample distribution matrix is designed to additionally serve as a reservoir, if needed.

The HDL test pad contains reagents for quantification of HDL-associated cholesterol. Preferably, these include cholesterol esterase, for releasing free cholesterol from HDL, cholesterol oxidase, for producing H_2O_2 by reaction with free cholesterol, peroxidase, and a coupled dye system which is converted, in the presence of peroxidase and H_2O_2, to a distinctively colored signal reaction product. The test pad may also comprise a biosensor effective to electrochemically quantify H_2O_2 and/or O_2, as described above.

The remaining test pads **66**, **68**, and **70** also contain assay reagents which produce a change in the pad, which can be detected optically, either visually or by a detector, in a known manner. In preferred embodiments of the current device and method, the non-HDL binding reagents are located in reagent pad **74**, and not in the sample distribution array or sieving pad. In this embodiment, the possibility of interference from these reagents, in assays of analytes other than HDL, is eliminated.

Preferably, each of the test pads contains reagent components for producing H_2O_2 via reaction of the analyte with an enzyme; the H_2O_2 subsequently converts a substrate reagent to a colored signal reaction product, or is measured electrochemically, as described above. Enzymatic color reactions which employ a variety of substrate-specific oxidases, for enzymatic generation of H_2O_2, and subsequent oxidation of a dye to form a colored reaction product, are well known.

A device having four or more test pads can be used to simultaneously measure HDL cholesterol (HDL), glucose, total cholesterol (TC), triglyceride lipid (TRG), ALT, AST, BUN, and/or creatinine. Each pad contains the above-

described common pathway components (peroxidase and a coupled dye system) such that generated H_2O_2 produces a distinctly colored signal reaction product. The total cholesterol test pad, which is exposed to serum without exposure to a precipitating or binding reagent, and the HDL test pads each include, in addition to the common pathway components, cholesterol esterase, for releasing esterified cholesterol in free-cholesterol form from serum lipoproteins, including HDL, LDL, and VLDL particles, and cholesterol oxidase, for producing H_2O_2 by reaction with free cholesterol in the sample fluid, as described above. The glucose assay pad includes glucose oxidase, in addition to the common-pathway components. The triglyceride pad includes, in addition to the common-pathway components, lipase, L-glycerol kinase, and L-glycerol-3-phosphate oxidase, for generating H_2O_2 from triglyceride, via the intermediate L-glycerol-3-phosphate. The serum sample drawn into the TRG pad is not exposed to precipitating or binding reagents, and thus contains all of the serum lipoproteins, so the TRG signal represents total serum triglycerides.

Reference standard pads may also be employed; see, for example, the system described in co-owned U.S. Patent No. 5,114,350, which is incorporated herein by reference.

EXAMPLES

The following examples illustrate but are in no way intended to limit the invention.

Example 1: Preparation of HDL Test Pad

A BTS-83 polysulfone membrane was loaded with HDL reagent at a loading volume of 77 µl / in2 and dried for 20 minutes at 50°C in a continuous roll process. Test pads containing the following HDL reagents were prepared: cholesterol oxidase 36.5 Units/ml, cholesterol esterase 215 Units/ml, peroxidase 200 Units/ml, 4-aminoantipyrine 1.88 mg/ml, and TOOS (3-[ethyl(3-methylphenyl)amino]-2-hydroxy propanesulfonic acid) 12.05 mg/ml. Lengths of *e.g.* 100 feet can be prepared in this manner and cut to fit the assay devices.

Example 2: Preparation of Reagent Pad with Binding Reagent

An aqueous reagent solution containing EAA dispersion at a concentration of 5.5% was applied to BTS-83 polysulfone membrane in a dip tank and dried for 20 minutes at 50° C in a continuous roll process. Next, an aqueous reagent solution containing 1 – 5 mg/ml dextran sulfate (500,000 MW) and 35 mM $Mg(OAc)_2$ was dispensed onto the same membrane using a syringe pump to meter reagent onto one surface of the membrane. Lengths of *e.g.* 100 feet can be prepared in this manner and cut to fit assay devices.

Example 3: Preparation of Assay Pad

A HDL test pad and a reagent pad were prepared according to Examples 1 and 2, respectively. The pads were oriented with the layers having the shiny side of the reagent pad (small pore size) contacting the dull or open pore side of the HDL test pad. The two membranes were passed through a lamination machine that heated the membranes to 80° C for 20 seconds in a continuous roll process.

Example 4: Representative Assay Procedure

A typical assay was carried out in an LDX® analyzer, using an assay pad, prepared essentially as described in Example 3. Sections of the adhered pads were hand cut to reaction membrane size and attached to the reaction bar. Alternatively, laminated assay pads can be applied to reaction bars in a continuous roll process using ultrasonic welding. A serum sample was loaded in the sample well. After sample distribution, the reaction bar was contacted with the adhered pads for approximately 4 seconds, a time sufficient to transfer enough serum to fill the HDL test pad, after which the bar was returned to its original position. The color was allowed to develop for 3 minutes and the reflectance was monitored by the LDX® analyzer.

Example 5: Acrylic Acid Copolymer Concentration on Reagent Pad

The concentration of EAA on the reagent pad was varied to determine porosity of the membrane. The reagent pad was prepared essentially according to Example 2. Serial dilutions of EAA were applied to the reagent pad starting with a 20%

solution. The reagent pads were adhered according to Example 3 to a HDL test pad. The dull side of the reagent pad was tested with a dilute aqueous solution of Evan's blue to determine penetration and/or diffusion of the colored solution through the reagent pad and into the HDL test pad. The highest concentration of EAA that allowed ready penetration and/or diffusion of the solution was about 10% EAA.

Example 6: Preparation of Assay Pad Using Heat

Two layers of BTS 83, without any reagents applied, are laminated together in a continuos roll process. The orientation of membranes is as described for Example 3. An aqueous solution of Dextran sulfate (50,000 MW) and $Mg(OAc)_2$ is coated onto the open pore side of the laminate using a standard gravure coater. The gravure coater is selected to dispense a non-saturating volume of reagent onto a single layer of the laminate. Following drying, the HDL reagent is applied to the opposite membrane in a similar manner. The reagents are concentrated from the levels shown in Example 1 and 2 to compensate for the reduced volume being applied.